

10/587956
IAP11 Rec'd PCT/PTO 31 JUL 2006

PCT/KR2004/001819
RO/KR 08. 10. 2004

A NOVEL HANSENULA POLYMORPHA GENE CODING FOR ALPHA 1,6-
MANNOSYLTRANSFERASE AND PROCESS FOR THE PRODUCTION OF
RECOMBINANT GLYCOPROTEINS WITH HANSENULA POLYMORPHA MUTANT
STRAIN DEFICIENT IN THE SAME GENE

5 Technical Field

The present invention relates to a novel *Hansenula polymorpha* gene coding for α -1,6-mannosyltransferase initiating outer chain elongation, an *H. polymorpha* mutant strain having a deficiency in the gene, and a process for 10 producing a recombinant glycoprotein using such a mutant strain.

Background Art

Upon large-scale expression of therapeutic proteins, according to characteristics of host cells or target proteins, a target protein may vary in expression levels, 15 water solubility, expression sites, modification, and the like. Thus, the most suitable expression system for a target protein must be selected to establish an effective production system.

Most therapeutic proteins are glycoproteins where 20 oligosaccharides are covalently bonded to asparagine residues as they pass through the endoplasmic reticulum (ER) and Golgi apparatus (Jenkins et al., Nat. Biotechnol., 14, 975-9, 1996). The structure and kind of sugar moieties

greatly affect folding, biological activity and stability in serum of glycoproteins. Thus, to date, for producing therapeutic recombinant glycoproteins having wild-type sugar moieties and therapeutic activity, the most commonly used 5 approach is to use animal cell expression systems. However, there are drawbacks to animal cell culture systems, which include low yield, high cost due to expensive culture media, retroviral contamination, and a long period of time required for establishing stable cell lines. Thus, animal cell 10 culture systems have limited applications in producing recombinant glycoproteins. In this regard, many attempts have been made to use, as an alternative to animal cell expression systems, yeast expression systems, which are eukaryotes and share the early steps of the N-linked 15 glycosylation pathway of higher animal cells, to produce recombinant glycoproteins of medical importance.

Eukaryotes such as yeasts have advantages of rapidly producing high-yield proteins, utilizing sterilized and well-controlled production conditions, being easily 20 genetically engineered, having no risk of infections by human or animal pathogens, and ensuring easy protein recovery. However, a complete type synthesized in yeasts has a different sugar moiety from that of target organisms such as mammals, and thus may cause immune responses in 25 animal cells. Also, this yeast-specific outer chain glycosylation of the high mannose type, also denoted hyperglycosylation, brings rise to heterogeneity of a recombinant protein product, which may make the protein

purification complicated or difficult. Further, the specific activity of enzymes may be lowered due to the increased carbohydrate level (Bekkers et al., Biochem. Biophys. Acta. 1089, 345-351, 1991).

5 To solve the above problems, there is a need for glycotechnology which introduces into yeasts a glycosylation pathway of animal cells capable of producing glycoproteins having identical biological activity to those derived from mammals.

10 When recombinant glycoproteins are expressed in traditional yeast, *Saccharomyces cerevisiae*, the addition of a series of 50 to 200 mannose residues to a core oligosaccharide, resulting in hypermannosylation, and the presence of α -1,3-linked terminal mannose recognizable as an antigen in the body were viewed as large constraints in employing the yeast as a host for glycoprotein production (Dean, Biochim. Biophys. Acta., 1426, 309-322, 1999; Ballou, Methods Enzymol., 185, 440-444, (1990)). By contrast, when recombinant glycoproteins are expressed in the methylotrophic yeasts, *Hansenula polymorpha* and *Pichia pastoris*, they are expressed in a hypermannosylated form compared to natural forms, but the overall length of mannose outer chains is shorter than those expressed in *S. cerevisiae* (Kang et al., Yeast 14, 371-381, 1998; Kim et al., Glycobiology, in press, 2004; Bretthauer and Castellino, Biotechnol. Appl. Biochem. 30, 193-200, 1999). In particular, since sugar chains synthesized in the methylotrophic yeasts, *H. polymorpha* and

P. pastoris, do not contain the strongly immunogenic α -1,3-linked terminal mannose (Kim et al., Glycobiology, in press, 2004; Montesino et al., Protein Expr. Purif. 14, 197-207, 1998), the methylotrophic yeasts are considered superior host systems to traditional yeast, *S. cerevisiae*, for the production of glycoproteins having therapeutic value in humans.

Many attempts were made in the glycotechnology field to develop hosts capable of producing therapeutic recombinant glycoproteins containing human compatible sugar chains using *P. pastoris* and *S. cerevisiae* (Chiba et al., J. Biol. Chem., 273, 26298-26304, 1998; Callewaert et al., FEBS Lett., 503, 173-178, 2001; Choi et al., Proc. Natl. Acad. Sci. U S A, 100, 5022-5027, 2003; Hamilton et al., Science, 301, 1244-1246, 2003). For example, an attempt was made to produce a glycoprotein where an intermediate including the human mannose-type $\text{Man}_5\text{GlcNAc}_2$ N-glycan was attached using a recombinant *S. cerevisiae* obtained by further genetically manipulating a triple mutant yeast (*och1Δmnn1Δmnn4Δ*) to express mammalian α -1,2-mannosidase in the ER (Chiba et al., J. Biol. Chem., 273, 26298-26304, 1998). The triple mutant has disruption in three genes: *OCH1* that plays a critical role in outer chain initiation (Nakanishi-Shindo et al., J. Biol. Chem. 268, 26338-26345, 1993; US Patent 5,705,616; US Patent 5,798,226); *MNN1* that mediates addition of the immunogenic α -1,3-linked terminal mannose (Gopal and Ballou, Proc. Natl. Acad. Sci. USA 84, 8824, (1987); US Patent 5,135,854); and *MNN4* that adds phosphates to a sugar chain

(Jigami and Odani, *Biochim. Biophys. Acta.*, 1426, 335-345, 1999). In addition, according to recent studies (Choi et al., *Proc. Natl. Acad. Sci. U S A*, 100, 5022-5027, 2003; Hamilton et al., *Science*, 301, 1244-1246, 2003), host developments in *P. pastoris* were made to produce recombinant glycoproteins with the human complex-type N-glycan GlcNAc₂Man₃GlcNAc₂ by introducing five different enzymes derived from eukaryotes into a secretory pathway in order to introduce the human glycosylation pathway into mutant strains 5 (Japanese Pat. 07145005; Japanese Pat. 07150780; International Pat. Publication WO 0200856 A2; International Pat. Publication WO 0200879 A2) which have a disruption in the *OCH1* gene mediating outer chain initiation. However, to date, from the viewpoint of glycotechnology, attempts have 10 rarely been made to produce recombinant glycoproteins with human-type sugar chains in the methylotropic yeast *H. polymorpha* which is gaining popularity as a host for the expression of therapeutic recombinant proteins since it has 15 been employed for producing hepatitis vaccines.

20 As described in Korean Pat. Application No. 2002-37717, the present inventors, before the present invention, cloned *OCH1* gene playing a critical role in the outer chain synthesis of *H. polymorpha*, establishing a mutant strain (*Hpoch1Δ*) having a disrupted *OCH1* gene, and developed a 25 process for producing a recombinant glycoprotein with a sugar chain structure closer to a natural form by preventing hyperglycosylation using such a mutant. However, in the

Hpoch1Δ mutant strain having a disruption in the *OCH1* gene of *H. polymorpha*, outer chain glycosylation is still initiated by α -1,6-mannose linkage. Thus, there is a need for the finding of a gene coding for α -1,6-mannosyltransferase and prevention of the above human incompatible glycosylation pathway.

Disclosure of the Invention

With an aim to overcome the above problems and develop a production system for a recombinant glycoprotein having therapeutic value in humans using a methylotrophic, thermotolerant yeast *Hansenula polymorpha* that is widely used as a host system for mass expression of various heterogeneous genes, the present inventors cloned a novel gene *HpOCH2* in a *H. polymorpha* strain DL-1 based on the known genome information for *H. polymorpha*, identified that the novel gene has the activity of α -1,6-mannosyltransferase responsible for the outer chain initiation, and developed a mutant strain having a disruption in the above gene. Then, the present inventors found that the mutant strain prevents a human incompatible glycosylation pathway, and, when a heterogeneous sugar chain-modifying enzyme is expressed in the mutant strain, is capable of producing a recombinant glycoprotein with a human mannose-type N-glycan $\text{Man}_5\text{GlcNAc}_2$ other than a yeast-specific N-glycan, thereby leading to the present invention.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows a nucleotide sequence of *H. polymorpha* HpOCH2 gene and its predicted amino acid sequence, wherein a transmembrane spanning region is underlined, and an amino acid sequence corresponding to a DXD element is bold and underlined;

Fig. 2 is a multiple alignment of amino acid sequences of Och1 protein analogues of *H. polymorpha* and other yeast strains (HpOch2p: *H. polymorpha* Och2 protein; HpOch1p: *H. polymorpha* Och1 protein; CaOch1p: *Candida albicans* Och1 protein; PpOch1p: *Pichia pastoris* Och1 protein; ScOch1p: *Saccharomyces cerevisiae* Och1 protein; and SpOch1p: *Schizosaccharomyces pombe* Och1 protein), wherein parenthesized numerals indicate amino acid identity between *H. polymorpha* HpOch2p and Och1p analogues of other yeast strains;

Fig. 3 is a diagram for inducing disruption of the *H. polymorpha* HpOCH2 gene by *in vivo* DNA recombination;

Fig. 4 shows growth properties of an *H. polymorpha* Hpoch2Δ mutant strain, wherein cultures ($OD_{600}=1$) of a *H. polymorpha* wild type and two mutant strains, Hpoch1Δ and Hpoch2Δ, which had arrived at an exponential phase, were 10-

fold serially diluted, and 3 µl of each dilution was spotted on a YPD medium and cultured for two days (A: YPD medium at 37°C; B: YPD medium at 45°C; C: YPD medium supplemented with 40 µg/ml hygromycine B; D: YPD medium supplemented with 0.4% sodium deoxycholate; and E: YPD medium supplemented with 7 µg/ml Calcofluor white);

Fig. 5 shows results of HPLC analysis for size distribution and structure of sugar chains attached to glucose oxidase (GOD) expressed in an *H. polymorpha* *Hpoch2Δ* mutant strain, wherein the left panel (A, B and C) and right panel (D, E and F) represent results for sugar chains attached to GOD expressed respectively in an *H. polymorpha* wild type and the *Hpoch2Δ* mutant (A and D: sugar chain profiles attached to GOD; B and E: sugar chain profiles after treatment with α-1,2-mannosidase; and C and F: sugar chain profiles after subsequent treatment with α-1,6-mannosidase), and retention times of standard oligosaccharides of known size and structure are indicated by arrows (M5: Man₅GlcNAc₂-PA; M6: Man₆GlcNAc₂-PA; M8: Man₈GlcNAc₂-PA; and M11: Man₁₁GlcNAc₂-PA);

Fig. 6 shows results of tests for functional compensation of a *S. cerevisiae* *och1Δ* mutant by introduction of *HpOCH2* gene (1: *S. cerevisiae* wild type transformed with a control vector YEpl352GAPII; 2: *S. cerevisiae* *och1Δ* mutant (*Scoch1Δ*) transformed with the control vector; and 3, 4 and 5: *Scoch1Δ* mutant transformed respectively with an *HpOCH1* gene expression vector YEpl352GAPII-*HpOCH1* (3), an *HpOCH2* gene expression vector YEpl352GAPII-*HpOCH2* (4), and a *ScOCH1* gene

expression vector YE_p352GAPII-ScOCH1(5)), wherein yeast cultures (OD₆₀₀=1) which had arrived at an exponential phase were 10-fold serially diluted, and 3 µl of each dilution was spotted on a YPD plate and cultured at 25°C and 30°C for 5 three days (the A of Fig. 6); and glycosylation of invertase expressed in each transformant was detected by activity staining (the B of Fig. 6);

Fig. 7 shows results of tests for measuring α-1,6-mannosyltransferase activity of *HpoCh2* protein, wherein a *S. cerevisiae och1Δmnn1Δmnn4Δ* mutant strain was transformed with a control vector YE_p352GAPII (A), an *HpoCH2* gene expression vector YE_p352GAPII-*HpoCH2* (B) or a *ScOCH1* gene expression vector YE_p352GAPII-*ScOCH1* (C), and membrane fractions obtained from the mutant strain were reacted with 10 Man₉GlcNAc₂-PA at 30°C for two hours and then analyzed by 15 HPLC;

Fig. 8 shows results of HPLC analysis for size distribution and structure of sugar chains attached to GOD expressed in *H. polymorpha* strains
20 (A and D: sugar chain profiles of GOD expressed in a secretory form respectively in a *H. polymorpha* wild type and an *HpoCh2Δ* mutant; B and E: sugar chain profiles of GOD expressed respectively in a *H. polymorpha* wild type and an *HpoCh2Δ* mutant which are genetically engineered to express 25 α-1,2-mannosidase derived from *Aspergillus saitoi*; and C and F: sugar chain profiles after treatment with α-1,2-mannosidase for sugar chains respectively from the recombinant wild type and mutant); and

Fig. 9 shows amino acid sequence identity and similarity between Och1 protein homologues of *S. cerevisiae* and *H. polymorpha*.

Best Mode for Carrying Out the Invention

5 In eukaryotes, protein N-glycosylation originates in the endoplasmic reticulum (ER), where an N-linked oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) is transferred to an appropriate asparagine residue of a nascent protein. From $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, three glucose residues and one specific α -1,2-linked mannose residue are removed by specific glucosidases and α -1,2-mannosidase in the ER, resulting in the core oligosaccharide structure, $\text{Man}_8\text{GlcNAc}_2$. The protein with this core sugar structure is transported to the Golgi apparatus where the sugar moiety undergoes various modifications by various specific enzymes. In yeasts, the modification of the sugar chain in the Golgi apparatus involves a series of additions of mannose residues by different mannosyltransferases. The structure of the outer chain glycosylation is specific to the organisms, typically with more than 50 mannose residues in *S. cerevisiae*.

10 15 20

The present inventors cloned an *HpOCH2* gene in a *H. polymorpha* strain DL-1 based on the known genome information for *H. polymorpha*, and identified that the above gene has the activity of α -1,6-mannosyltransferase responsible for the outer chain initiation. The identified gene has a nucleotide sequence designated as SEQ ID NO. 1, and its corresponding

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amino acid sequence is designated as SEQ ID NO. 2.

In one aspect, the present invention provides a DNA gene coding for a protein designated as SEQ ID NO. 2. In addition, the present invention provides a DNA gene coding for a protein having a 75% or higher homology with the former DNA and coding for a protein with α -1,6-mannosyltransferase enzyme activity. Preferably, the present invention provides a gene having a DNA sequence designated as SEQ ID NO. 1, an analogue thereof or a fragment thereof.

The term "homology", as used for a α -1,6-mannosyltransferase gene derived from *H. polymorpha* in the present invention, is intended to indicate the degree of similarity to the nucleotide sequence of a wild type, and includes a DNA sequence having an identity of preferably 75% or higher, more preferably 85% or higher, and most preferably 90% or higher, with a DNA sequence coding for α -1,6-mannosyltransferase. This homology comparison may be performed manually or by using a commercially available comparison program. A commercially available computer program may express homology between two or more sequences as a percentage, and a homology (%) may be calculated for adjacent sequences.

The present inventors registered the above gene at GenBank under accession number AY502025, and deposited a recombinant vector containing the gene, pBS-HpOCH2/*Escherichia coli* DH5 α , at KCTC (Korean Collection for Type Cultures; KRIBB, 52, Oun-dong, Yusong-ku, Taejon, Korea) on January 15, 2004, under accession number KCTC 10583BP.

Thus, in another aspect, the present invention provides a recombinant vector comprising a gene which is DNA coding for a protein designated as SEQ ID NO. 2 or DNA having a 90% or higher homology with the former DNA and 5 coding for a protein with α -1,6-mannosyltransferase activity. The recombinant vector preferably comprises a DNA gene designated as SEQ ID NO. 1. In a further aspect, the present invention provides a host cell transformed with the recombinant vector, and preferably, provides a transformed 10 host cell deposited under accession number KCTC 10583BP.

To produce a glycoprotein having a mammalian-type sugar chain in a yeast, a mutant strain yeast should be established, which lacks an enzyme family involved in yeast outer chain biosynthesis. Such a mutant strain may be 15 attained by genetic mutation such as use of a reagent, ultraviolet illumination or spontaneous mutation, or by artificially disrupting a target gene. In the present invention, a gene (*Och2*) encoding α -1,6-mannosyltransferase playing a critical role in the outer chain initiation is 20 disrupted by genetic engineering methods, that is, a combination of polymerase chain reaction and *in vivo* DNA recombination.

The present inventors established a *Hansenula polymorpha* *Hpoch2 Δ* mutant strain (*Hansenula polymorpha* DL-1 25 *och2 Δ*) in which a α -1,6-mannosyltransferase gene identified as described above is deficient, and found that yeast-specific consecutive addition of α -1,6-mannose residues is prevented in the mutant strain, so that hyperglycosylation is

remarkably reduced. The mutant strain was deposited at KCTC (Korean Collection for Type Cultures; KRIBB, 52, Oun-dong, Yusong-ku, Taejon, Korea) on January 15, 2004, under accession number KCTC 10584BP.

5 Thus, in yet another aspect, the present invention provides a *Hansenula polymorpha* *Hpoch2Δ* mutant strain (*Hansenula polymorpha* DL-1 och2Δ) deposited under accession number KCTC 10584BP.

The majority of N-glycans on glycoproteins transported from the ER have a Man₈GlcNAc₂ sugar chain structure. After a protein is transported to the Golgi apparatus from the ER, additional mannose residues are added to the protein by different mannosyltransferases, resulting in a glycoprotein having numerous mannose sugar chains. The hyperglycosylation is undesirable in recombinant glycoproteins. Such hyperglycosylation may be reduced by using the *H. polymorpha* mutant strain prepared in the present invention as a host cell for the expression of recombinant proteins. In addition, when the *H. polymorpha* mutant strain is transformed with an expression vector capable of expressing one or more proteins having an enzymatic activity involved in sugar chain modifications, the hyperglycosylation may be more effectively inhibited or be converted to a sugar chain with a different structure. Sugar chain-modifying enzymes involved in such hyperglycosylation reduction include α-1,2-mannosidase, mannosidase IA, mannosidase IB, mannosidase IC, mannosidase II, N-acetyl glucosaminyltransferase I, N-acetyl glucosaminyltransferase

II, galactosyltransferase, sialyltransferase, and fucosyltransferase. However, the present invention is not limited to the above examples, and various genes capable of leading to a reduction and modification in hyperglycosylation of a recombinant glycoprotein may be also used. In an embodiment of the present invention, when α -1,2-mannosidase was expressed in the *H. polymorpha* Hpoch2 Δ mutant strain, a recombinant glycoprotein on which a yeast-type N-glycan was prevented from being formed and modified to a human-type N-glycan was produced. α -1,2-mannosidase removes a α -1,2-linked mannose residue at a non-reduced terminal of Man₈GlcNAc₂ and converts a core sugar chain on this glycoprotein to Man₅GlcNAc₂. The Man₅GlcNAc₂ structure is an inferior substrate for Golgi-residing mannosyltransferases, leading to a glycoprotein having reduced mannose content. The sugar chain-modifying enzyme gene contained in the expression vector used in the transformation may be the whole gene sequence encoding such an enzyme or a fragment sequence encoding a functional region of the enzyme. The expression vector includes an integrative or inductive promoter and a 3' termination sequence, and may be an integrative or replicative vector.

Thus, in still another aspect, the present invention provides a *H. polymorpha* mutant strain further comprising an expression vector expressing a sugar chain-modifying enzyme. Preferably, the sugar chain-modifying enzyme is selected from the group consisting of α -1,2-mannosidase, mannosidase IA, mannosidase IB, mannosidase IC, mannosidase II, N-acetyl

glucosaminyltransferase I, N-acetyl glucosaminyltransferase II, galactosyltransferase, sialyltransferase, and fucosyltransferase.

In still another aspect, the present invention
5 provides a process for producing a recombinant glycoprotein with reduced glycosylation using an *H. polymorpha* mutant strain deposited under accession number KCTC 10584BP.

According to the present process as described above, a recombinant glycoprotein may be produced in a manner such
10 that formation of a yeast-type N-glycan is prevented and the yeast-type N-glycan is modified to a human-type N-glycan.

The term "glycoprotein", as used herein, refers to a protein that is glycosylated on one or more asparagines, or one or more serine or threonine residues, or is glycosylated
15 on asparagine and serine or threonine residues when expressed in a methylotrophic yeast, particularly *Hansenula polymorpha*. The term "reduced glycosylation", as used herein, means that, when a glycoprotein is expressed in a methylotrophic yeast strain, it has a reduced size of a carbohydrate moiety, particularly lower mannose residues, in comparison with the case of being expressed in a wild-type methylotrophic yeast.
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In the above process, a glycoprotein expression vector introduced into the *Hansenula polymorpha* *Hpcho2Δ* mutant strain preferably expresses a sugar chain-modifying enzyme such as α-1,2-mannosidase along with glycoprotein.
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A produced glycoprotein may be purified by a method commonly used in the art, and a purification protocol may be determined according to the properties of a specific protein

to be purified. This determination is considered an ordinary skill to those skilled in the art. For example, a target protein may be purified by a typical isolation technique, such as precipitation, immunoadsorption, fractionization or various chromatographic methods.

Glycoproteins capable of being produced according to the present invention are exemplified by cytokines (e.g., interferon- α , interferon- β , interferon- γ , G-CSF, etc.), clotting factors (e.g., VIII factor, IX factor, human protein C), endothelial growth factor, growth hormone releasing factor, *Penicillium minioluteum* dextranase, *Bacillus amyloliquefaciens* α -amylase, *Saccharomyces cerevisiae* aspartic protease, *Saccharomyces cerevisiae* invertase, *Typanosoma cruzi* trans-sialidase, HIV envelope protein, influenza virus A haemagglutinin, influenza neuraminidase, bovine enterokinase activator, bovine herpes virus type-1 glycoprotein D, human angiostatin, human B7-1, B7-2 and B-7 receptor CTLA-4, human tissue factor, growth factors (e.g., platelet-derived growth factor), human α -antitrypsin, human erythropoietin, tissue plasminogen activator, plasminogen activator inhibitor-1, urokinase, α -galactosidase, plasminogen, thrombin, and immunoglobulins.

In still another aspect, the present invention provides a glycoprotein produced by the above process.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Identification of *Hansenula polymorpha* *HpOCH2* gene and analysis of the amino acid sequence of the gene

From the recently completed sequence of *Hansenula polymorpha* RB11 genome (Ramezani-Rad et al., FEMS Yeast Res., 4, 207-215 (2003)), whole sequences of ORFs (open reading frames) having a high similarity with the *OCH1* gene family involved in outer chain biosynthesis of *Saccharomyces cerevisiae* were obtained. Fig. 9 shows amino acid sequence identity and similarity between Och1 protein homologues of *H. polymorpha* and *S. cerevisiae*, wherein the amino acid sequence identity and similarity between ORF168 and ScOch1p are represented by shaded bold numerals.

In the present invention, for functional analysis of the ORF168 gene having a 40% amino acid identity and a 54% amino acid similarity with *S. cerevisiae* *OCH1* gene (ScOCH1) (Jungman and Munro, Embo J. 17, 423 (1998)) that plays a critical role in α -1,6-mannose addition at the early stage of outer chain biosynthesis of *S. cerevisiae*, polymerase chain reaction (PCR) was carried out using DNA extracted from *Hansenula polymorpha* DL-1 (Levine and Cooney, Appl. Microbiol., 26, 982-990, (1973)) as a template and a pair of primers(168Not-N and 168Not-C; Table 1). As a result, a 1.35-kb DNA fragment containing the ORF168 was obtained, and was then subjected to amino acid sequencing.

A conventionally identified *H. polymorpha* gene described in Korean Pat. Application No. 2002-37717 applied

by the present inventors has an amino acid sequence having a 22% identity and a 36% similarity with *S. cerevisiae OCH1* gene and is thus designated as *HpOCH1*. In this regard, the ORF168 identified in this invention was designated as *HpOCH2*,
5 and its nucleotide sequence was registered at GenBank under accession number AY502025. *HpOCH2* was 1287 bp long and expected to code for a protein consisting of 428 amino acids. *HpOch2* protein had a potential transmembrane spanning region at a region from 29 to 51 positions, and was thus considered
10 as a type II membrane protein to which most glycosyltransferases belong (Fig. 1). Also, *HpOch2* protein was observed to have a DXD element known as an active site of glycosyltransferases (Lussier et al., J. Cell. Biol., 131, 913-927, (1995)), and was thus expected to have
15 glycosyltransferase activity (Fig. 1). The amino acid sequence of *HpOch2* protein was found to have a relatively high similarity with, in addition to a *S. cerevisiae OCH1* gene product, *OCH1* gene products of other yeasts, that is, *Candida albicans*: Thomas et al., unpublished results, GenBank accession number AY064420), *Pichia pastoris*: Japanese Pat.
20 07145005), *Schizosaccharomyces pombe*: Yoko-o et al., FEBS Lett., 489, 75-80, (2001)) (Fig. 2).

EXAMPLE 2: Establishment of *H. polymorpha HpOCH2* gene-deficient strain and analysis of characteristics of the
25 strain

To establish a *H. polymorpha HpOCH2* gene-deficient

mutant strain, gene disruption was performed by a combination of fusion PCR and *in vivo* DNA recombination (Oldenburg et al., Nucleic Acid Res., 25, 451, (1997)). Fusion PCR was carried out using primers (primers used for PCR for cloning and disruption of *HpOCH2* gene) listed in Table 1, below. By primary PCR, 5' and 3' regions of *URA3* gene and *HpOCH2* gene were obtained. By secondary fusion PCR, the 5' region of *HpOCH2* gene was linked to the 5' region of *URA3* gene, and the 3' region of *URA3* gene was linked to the 3' region of *HpOCH2* gene. Then, the two DNA fragments were introduced into a yeast cell, and transformants having an *HpOCH2* gene disrupted by *in vivo* DNA recombination were selected (Fig. 3). Primarily, using an *URA3* selection marker, transformants grown in a minimum medium lacking uracil were selected. Then, amplified DNA fragments produced by PCR were examined to determine whether they differ from those of a wild-type strain, thereby selecting a *H. polymorpha* mutant strain having a different amplification pattern, *Hpoch2Δ* (*leu2 och1::URA3*). The obtained *Hpoch2Δ* strain was evaluated for growth properties. The *Hpoch2Δ* strain was found to have temperature sensitivity at 45°C like a *Hpoch1Δ* strain (KCTC 10264BP), but, unlike the *Hpoch1Δ* strain, had a similar growth rate to the wild type at 37°C. Also, growth was greatly inhibited in the presence of hygromycin B, and little sensitivity to sodium deoxycholate was observed (Fig. 4). Since these growth properties are common in mutant strains having a defect in outer chain synthesis, the *H. polymorpha*

Hpoch2Δ strain was believed to have a defect in the outer chain glycosylation process.

TABLE 1

Primer	Sequences	SEQ ID. No.
168Not-N	5' -AAGGAAAAAAAGCGGCCGCGGTGAAGAATGGTGTAT-3'	3
168Not-C	5' -TTTCCTTGCAGCCGCGTCTGTGCCTGCTCATGAT-3'	4
UNfor	5' -GGATCCCCGGGTACCGAGCT-3' ^a	5
UNrew	5' -CACCGGTAGCTAATGATCCC-3'	6
UCfor	5' -CGAACATCCAAGTGGCCGA-3'	7
UCrew	5' -CTGGCGAAAGGGGGATGTGC-3' ^b	8
168Nfor	5' -GGCGGATATGGGGCTTCGCC-3'	9
168Nrew	5' -AGCTCCGGTACCCCCGGCACTCCCGTCCAGGGCTCCACGTCC-3' ^c	10
168Cfor	5' -GCACATCCCCCTTTCGGCCAGCCGATCACGAGCTTCAGTCC-3' ^d	11
168Crew	5' -CGTCGTCCGGGCCAGTCG-3'	12

EXAMPLE 3: Analysis of size distribution and structure of
5 sugar chains on a glycoprotein synthesized in the *H.*
polymorpha *Hpoch2Δ* mutant

To analyze the size distribution and structure of sugar chains on glycoprotein synthesized in the *H.* *polymorpha* *Hpoch2Δ* mutant prepared in Example 2, a 10 glycoprotein derived from *Aspergillus niger*, glucose oxidase (GOD), was expressed in a secreted form in a *H. polymorpha* wild type and the *Hpoch2Δ* mutant. The glycoprotein, GOD, has eight putative amino acid sequences for N-linked glycosylation (Frederick et al., J. Biol.Chem., 265, 3793 15 (1990)). The *H. polymorpha* wild type and *Hpoch2Δ* mutant

were individually transformed with an expression vector pDLMOX-GOD(H) expressing GOD with a six-histidine tag (Kim et al., Glycobiology (2004)), and were grown in YPM medium (1% yeast extract, 2% peptone, 2% methanol) supplemented with 2% methanol to express GOD. GOD secreted to the culture medium was passed through a nickel column to selectively isolate only GOD tagged with six histidines at the C-terminal region. The isolated recombinant his-tagged GOD was treated with PNGase F to detach attached sugar chains from the GOD. Then, the released sugar chains were labelled with 2-aminopyridine (2-PA) and subjected to HPLC analysis. As shown in the A and D of Fig. 5, sugar chains of wild type-derived recombinant GOD were found to have various size distributions ranging from 8 to 12 mannose residues. In contrast, sugar chains attached to recombinant GOD expressed in the *Hpoch2Δ* mutant were found to mostly have core sugar chains with 8 mannose residues. These results indicate that sugar addition after the eighth mannose residue is greatly inhibited in the *Hpoch2Δ* mutant. Separately, the sugar chains released from recombinant GOD were treated sequentially by α-1,2-mannosidase and α-1,6-mannosidase to investigate changes in a sugar chain profile. Sugar chains synthesized by the wild type were converted to sugar chains corresponding to five or six mannose, and all of them were then converted to sugar chains corresponding to five mannose by α-1,6-mannosidase, whereas all sugar chains of the *Hpoch2Δ* mutant were converted to sugar chains corresponding to five mannose by

only α -1,2-mannosidase (Fig. 5). These results reveal that initiation of outer chain elongation via α -1,6-mannose linkage never occurs in the *Hpoch2 Δ* mutant, thereby indicating that the *HpOCH2* gene product is directly or
5 indirectly involved in the activity of α -1,6-mannosidase.

EXAMPLE 4: Functional analysis of *H. polymorpha* *HpOCH2* protein

To determine whether the *H. polymorpha* *HpOCH2* gene product is a functional homologue to *S. cerevisiae* *Och1* protein adding α -1,6 mannose to a core sugar chain in the outer chain synthesis initiation process, an expression vector carrying an *HpOCH2* gene, YEp352GAPII-*HpOCH2*, was introduced into a mutant strain having a disruption in *S. cerevisiae* *OCH1* gene, *Scoch1 Δ* , and the *Scoch1 Δ* mutant was evaluated for ability to overcome thermosensitivity (the A of Fig. 6). *HpOCH2*, *HpOCH1* and *ScOCH1* genes were individually inserted between glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and terminator introduced into a *S. cerevisiae* expression vector YEp352 (Hill et al., Yeast, 2, 163-167, 10 1986)), thus generating expression vectors, YEp352GAPII-*HpOCH2*, YEp352GAPII-*HpOCH1* and YEp352GAPII-*ScOCH1*, respectively. The *S. cerevisiae* *Scoch1 Δ* mutant, when transformed with the *HpOCH2* gene expression vector (YEp352GAPII-*HpOCH2*), recovered its ability to grow at high 15 temperature. By contrast, when transformed with the expression vector (YEp352GAPII-*HpOCH1*) carrying *HpOCH1* gene
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having a nucleotide sequence similarity to *HpOCH2* gene, the *Scoch1Δ* mutant did not overcome thermosensitivity (the A of Fig. 6). In addition, the *Scoch1Δ* mutant was evaluated for another feature of having a defect in hyperglycosylation.

5 When the *HpOCH2* expression vector was introduced into the *Scoch1Δ* mutant, as shown in the B of Fig. 6, the glycosylation of a glycoprotein, invertase, recovered to a level identical to that when the *OCH1* expression vector (YEpl352GAPII-*ScOCH1*) was introduced thereinto. By contrast,

10 when the *HpOCH2* expression vector was introduced into the *Scoch1Δ* mutant, glycosylation of invertase was unchanged. The results, that the *S. cerevisiae och1Δ* mutant overcomes thermosensitivity and recovers hyperglycosylation by the expression of *H. polymorpha HpOCH2* gene, demonstrate that the

15 *HpOCH2* gene product is a functional analogue to *S. cerevisiae* Och1 protein playing a critical role in the first step of outer chain synthesis.

An *in vitro* assay was performed to determine whether the *H. polymorpha* HpOch2 protein practically has α-1,6-mannosyltransferase activity to add α-1,6-mannose to a core sugar chain like the *S. cerevisiae* Och1 protein. A mutant strain (*och1Δmnn1Δmnn4Δ*) disrupted in three genes, *OCH1*, *MNN1* and *MNN4*, has a complete loss of outer chain synthesis (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)).

20 The *och1Δmnn1Δmnn4Δ* mutant was transformed with the *H. polymorpha* *HpOCH2* gene expression vector, and a membrane fraction was prepared. The membrane fraction was used as an

enzyme source for measuring α -1,6-mannosyltransferase and reacted with a substrate having a core sugar chain structure, Man₉GlcNAc₂-PA. The resulting reaction solution was analyzed by HPLC. When the *och1Δmnn1Δmnn4Δ* mutant was transformed 5 with the Yep352GAPII vector not containing *HpOCH2* gene, the concentration of the substrate Man₉GlcNAc₂-PA was not changed in a membrane fraction. By contrast, when the *och1Δmnn1Δmnn4Δ* mutant was transformed respectively with the *H. polymorpha* *HpOCH2* gene expression vector and the *S. cerevisiae* *OCH1* gene expression vector, a peak corresponding 10 to Man₉GlcNAc₂-PA (a structure formed by the addition of a single mannose to Man₈GlcNAc₂-PA) was observed in membrane fractions (Fig. 7). These results indicate that the *H. polymorpha* HpOch2 protein, like the *S. cerevisiae* Och1 15 protein, has the activity of α -1,6-mannosyltransferase involved in the initiation of outer chain elongation.

EXAMPLE 5: Glycotechnology using the *H. polymorpha* *Hpoch2Δ* mutant

An *H. polymorpha* strain capable of producing a 20 recombinant glycoprotein having a human mannose-type N-linked glycan was established as follows. As described in a previous study (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)) carried out with the traditional yeast *Saccharomyces cerevisiae*, the *H. polymorpha* *Hpoch2Δ* mutant 25 was transfected with a α -1,2-mannosidase expression vector for application to *H. polymorpha*, pDUMOX-MsdS(HA-HDEL), in

order to express *Aspergillus saitoi* α -1,2-mannosidase in the ER of *H. polymorpha*, thereby developing a glycoengineered recombinant strain *Hpoch2Δ-MsdSp*. To construct the α -1,2-mannosidase expression vector, pDUMOX-MsdS(HA-HDEL), PCR was carried out using a plasmid containing *Aspergillus saitoi* α -1,2-mannosidase, pGAMH1 (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)), as a template and a forward primer (5'-GGGAAATTCAAAAAATGGTGGTCTTCAGCAAA-3': SEQ ID. NO. 13) containing an EcoRI site, and a reverse primer (5'-GGGCCATGGTCACAATTCATCATGCGCATAGTCAGGAACATCGTATGGGTATGTACTACTCACCCGCAC-3': SEQ ID. NO. 14) containing an HA sequence for determining protein expression levels, an HDEL (His-Asp-Glu-Leu) sequence as an endoplasmic reticulum retention/retrieval tag and a NcoI site. As a result, the *A. saitoi* α -1,2-mannosidase was amplified, thus yielding a 1.5-kb fragment. The 1.5-kb fragment was digested with EcoRI and NcoI and replaced a GOD gene of a GOD expression vector pDLMOX-GOD(H) (Kim et al.. Glycobiology, in press, (2003)). Then, in the resulting GOD expression vector, an *H. polymorpha* LEU2 selection marker was replaced by an *H. polymorpha* URA3 selection marker, thus finally yielding the α -1,2-mannosidase expression vector pDUMOX-MsdS(HA-HDEL). The expression of *A. saitoi* α -1,2-mannosidase in *H. polymorpha* was detected by Western blotting using an anti-HA antibody (Sigma).

To determine whether the glycoengineered *H. polymorpha* strain *Hpoch2Δ-MsdSp* synthesizes a human mannose-type *N*-glycan, the structure of sugar chains attached to GOD

expressed in a secreted form was analyzed. In a recombinant wild-type strain, *HpOCH2*-MsdSp, transformed with a heterogeneous α -1,2-mannosidase, sugar chains with 8 or higher mannose residues were sharply reduced, while sugar chains with 5 or 6 mannose residues were increased (the A and B of Fig. 8). In the recombinant *Hpoch2Δ*-MsdSp strain prepared by transforming the *Hpoch2Δ* strain with a heterogeneous α -1,2-mannosidase, sugar chains with more than 7 mannose residues were reduced in comparison with the recombinant wild-type strain *HpOCH2*-MsdSp (the B and E of Fig. 8). When sugar chains isolated from the recombinant wild-type strain and the *Hpoch2Δ* mutant strain were treated with α -1,2-mannosidase, the sugar chains of the *HpOCH2*-MsdSp strain were converted to sugar chains with 5 and 6 mannose residues, whereas sugar chains of the *Hpoch2Δ*-MsdSp strain were converted to sugar chains with 5 mannose residues by α -1,2-mannosidase (the C and F of Fig. 8). These results reveal that, when the heterogeneous α -1,2-mannosidase is introduced into *H. polymorpha*, a wild-type strain still forms yeast-specific α -1,6-mannose linkage by *H. polymorpha* HpOch2 protein, thereby indicating that, to synthesize human mannose-type N-glycans, *H. polymorpha* *HpOCH2* gene should be essentially disrupted. Therefore, the *Hpoch2Δ* mutant strain having a deficient *HpOCH2* gene, developed in the present invention, is useful as a host for the production of therapeutic recombinant glycoproteins having human compatible sugar chains. In addition, when various sugar chain-

modifying enzymes are expressed in the *Hpoch2Δ* mutant strain, they come to have various sugar moieties that are not immunogenic in the human body. Thus, the *Hpoch2Δ* mutant strain is very useful in glycotechnology for the development 5 of a host producing a novel glycoprotein having increased physiological activity as well as having non-immunogenic sugar moieties.

Industrial Applicability

Since *Hansenula polymorpha* has been approved 10 worldwide as a host system for mass production of recombinant hepatitis vaccines, recombinant proteins to be expressed in *H. polymorpha* have a high potential to be developed as biologics. As described in the above Examples, in the *Hpoch2Δ* mutant strain having a deficiency in *H. polymorpha* *HPOCH2* gene, developed in the present invention, 15 the initiation of outer chain elongation is prevented, resulting in the prevention of yeast-specific consecutive α-1,6-mannose addition. Thus, the *H. polymorpha* mutant strain can be used as a host to produce a target glycoprotein in the 20 form of having a sugar chain structure closer to that of human glycoproteins via a secretory pathway. Also, as described above, the *Hpoch2Δ* mutant strain becomes a basis in glycoengineering for the development of various *H. polymorpha* strains which may be used as hosts for the mass 25 production of recombinant glycoproteins of therapeutic value.

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Therefore, the present *Hpoch2Δ* mutant strain is very useful in related industrial fields.

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A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>11</u> , line <u>25-28</u>	
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Address of depositary institution(<i>including postal code and country</i>) #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	
Date of deposit January 15 2004	Accession Number KCTC 10583BP
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